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14. ABSTRACT

Polymer micelles are a nanoparticle drug delivery system that has the potential to improve breast tumor treatment with chemotherapy. These nanoparticles can increase the half-life of incorporated drugs, can target tumors by incorporating tumor specific ligands, and can be tracked with imaging through the inclusion of a radiolabel. In this study, PEG/PLA or PEG/PCL micelles were modified through the addition of a cRGD targeting ligand and a DOTA chelating molecule for tracking with 111In SPECT imaging. In vitro, cRGD-targeted micelles were incorporated into cells at a faster rate than non-targeted micelles and exhibited cytotoxicity rivaling that of free doxorubicin. Furthermore, SPECT imaging of 111In-labeled micelles administered intravenously to tumor-bearing mice in vivo established the potential for micelle tracking with 111In and confirmed the prolonged distribution half-life of micelles. Future research will focus on further development of this micelle platform with the ultimate goal of simultaneously tracking micelle localization and monitoring antitumor efficacy. Fluorescent tracking of micelles through the incorporation of quantum dots and monitoring antitumor efficacy with fluorescently labeled Annexin V may provide an alternate and superior method of achieving this goal.

15. SUBJECT TERMS
breast cancer, micelle, computed tomography (CT), single photon emission tomography (SPECT), doxorubicin, [111]In, [99m]Tc, passive targeting, apoptosis, annexin

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Table of Contents

Introduction and Summary

Polymer micelles are nano-sized aggregates of amphiphilic polymers that can be used as a delivery agent for breast cancer chemotherapy. Micellar drug formulations have a number of advantages over conventional chemotherapy. First, they increase the solubility of hydrophobic drugs, making them easier to administer intravenously[1]. Second, they have been shown to accumulate passively in tumors because of their leaky vasculature, which is known as the enhanced permeation and retention (EPR) effect[2]. Third, they have considerable potential for customization, in which additional functional groups can be attached to the surface and used to modulate micelle properties. Particularly, the addition of a targeting ligand can increase the specificity of drug delivery to tumors[3], and the attachment of a tracking molecule, such as a radiolabel, can facilitate tracking of micelles *in vivo*[4]. Previous work by our lab has established the fabrication of poly(ethylene glycol)/poly(ε-caprolactone) (PEG-PCL) micelles incorporating the anti-cancer agent doxorubicin[5]. These micelles have been further modified to include a cyclic RGD (cRGD) ligand to increase binding to endothelial cells[6] or to incorporate superparamagnetic iron oxide (SPIO), a T_2-specific MRI contrast agent to allow MRI tracking of micelles in vivo[7]. The goal of this project is develop a micelle platform for breast cancer treatment that can be tracked *in vivo* while simultaneously designing a method that can be used to assess the antitumor effects.

This report describes the accomplishments that have been achieved in pursuit of this overall goal. First, doxorubicin-loaded micelles modified with a cRGD ligand have been generated and evaluated in cells *in vitro*. Second, polymer micelles incorporating a 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) chelating group were fabricated and characterized. Finally, ^{111}In-labeled micelles were imaged *in vivo* using CT/SPECT in a preliminary attempt to monitor micelle distribution. Continuation of this work will include further refinement of *in vivo* micelle tracing and development of an imaging method to assess tumor response. The ultimate result of this project should be a system that uses imaging methods to track micelle accumulation and its antitumor effects on a breast cancer model in mice.

Body

The main goal of this project is to develop a micelle platform for breast cancer treatment and assess its effects in an animal model. We proposed to achieve this through the following three specific aims:

Aim 1: Producing doxorubicin-containing micelles that have an added chelating group for SPECT imaging.

Aim 2: Developing CT/SPECT imaging methodology for evaluating micelle treatment efficacy.

Aim 3: Using *in vivo* efficacy data to optimize the design of drug-loaded micelles and compare them directly to systemic chemotherapy for treating murine breast cancer.

The following section describes the progress that has been made in pursuit of each aim.

Aim 1

The goal of aim 1 was to develop drug-loaded micelles which incorporated a DOTA chelating ligand for ^{111}In labeling. To achieve this goal, we developed two populations of micelles, one incorporating a cRGD targeting ligand and a second incorporating both a cRGD ligand and DOTA chelator.

Poly(ethylene glycol)-poly(D,L-lactide) (PEG/PLA) micelles were fabricated and modified with the incorporation of a cRGD targeting ligand. Maleimide(MAL)-terminated PEG-PLA was synthesized by ring opening polymerization of D,L-lactide with MAL-PEG-OH as macro-initiator and $Sn(Oct)_2$ as a catalyst. Synthesized polymer was then characterized with NMR and gel permeation chromatography (GPC). The resulting polymer was MAL-terminated PEG (3.2 kD)/PLA (4.2 kD) with a molecular weight, M_n, of 7.2 kD. Micelles were fabricated using MAL-PEG-PLA along with monomethoxy-terminated PEG (2.0 kD)/ PLA (4.4 kD) (MPEG-PLA) to control the MAL density. Micelles were formed by adding a solution of doxorubicin/MAL-PEG-PLA/MPEG-PLA to aqueous solution under sonication. Micelles with a maleimide density of 0, 5, or 16% were generated. c(RGDf(*ε*-*S*-acetylthioacetyl)K and 0.05 M hydroxy amine in HEPES/EDTA aqueous solution were added into solutions of micelles with 5 and 16 maleimide density and reacted overnight. The resulting products were purified, and cRGD labeling verified with NMR. To test the cytotoxicity of these micelles, SLK tumor endothelial cells were incubated with micelles for 1 hour, and cytotoxicity was measured using a DNA assay 4 days after treatment. Relative cell viability is shown in Figure 1. These results indicate that micelles with the attached cRGD ligand are substantially more toxic than those without cRGD. An additional column (16% RGD + RGD) shows that the added toxicity due to the cRGD targeting can be reversed by adding free cRGD ligand to the media.

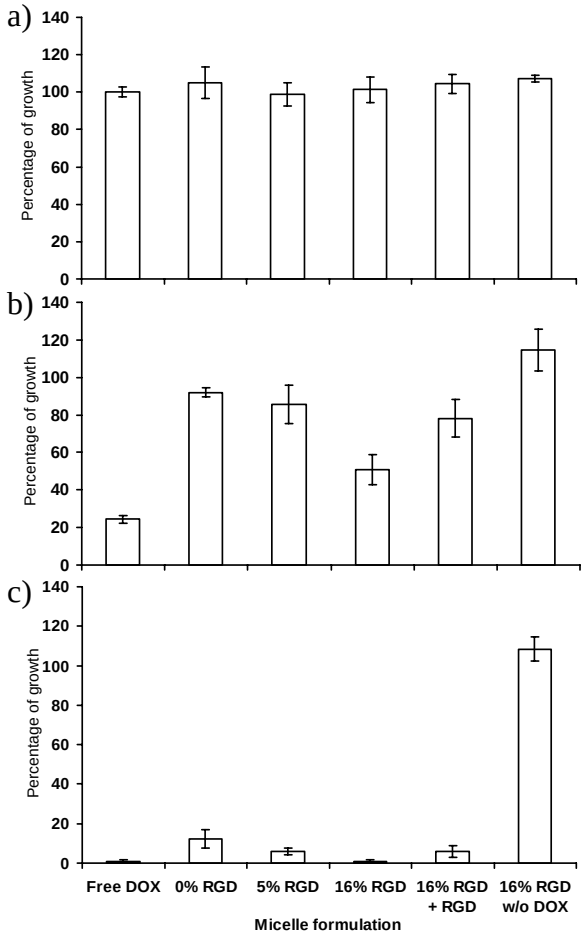

Figure 1. Percentage of growth in SLK cells for different formulation of SPIO-loaded micelles at a-c) 0.1, 1 and 10 μM DOXO concentration, 1 h coincubation. The percentage of growth inhibition was calculated as the ratio of cell numbers for treated samples over untreated control. Error bars were obtained from triplicate samples.

A second population of (PEG-PCL) micelles incorporating both a cRGD targeting ligand and DOTA chelating agent was fabricated for use in SPECT imaging. To generate this polymer, acetonitrile was reacted with ethylene oxide to form CN-PEG. To synthesize the block copolymer, CN-PEG was reacted with the exact amount of ε-caprolactone. The resulting CN-PEG-

PCL polymer was characterized by GPC and NMR. CN-PEG-PCL was then hydrogenated to form NH_2-PEG-PCL. Micelles were formed using the desired ratios of PEG-PCL, cRGD-PCL (synthesized as described previously), and CN-PEG-PCL, and doxorubicin. To attach the DOTA ligand, micelles were reacted with DOTA-NHS ester overnight, after which they were purified by dialysis. The resulting micelles contained 16% cRGD and 21.5% (by mole) DOTA incorporation. These micelles could then be labeled with ^{111}In for single photon emission computed tomography (SPECT) imaging.

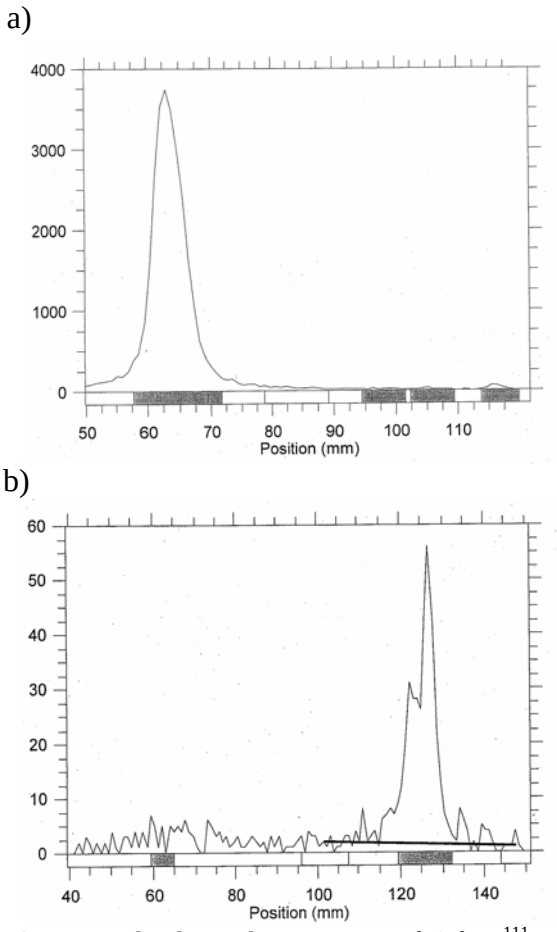

Figure 2. Thin layer chromatogram of a) free ^{111}In and b) ^{111}In/cRGD-labeled micelles.

Aim 2

This aim focuses on the development of methodology for imaging micelles with combined computed tomography (CT)/SPECT imaging. DOTA-containing micelles were radiolabeled to allow for *in vivo* micelle tracking. To add ^{111}In to the micelles, anhydrous $InCl_3$ in ammonium acetate buffer (0.5 M, pH 6.0) was added and the mixture heated to 40ºC for 70 minutes. Excess indium was removed through gel filtration. Indium labeling was verified by thin layer chromatography equipped with a radioactivity detector. The resulting chromatograph is shown in Figure 2. No free indium (57-72 mm) and only labeled micelles (112-135 mm) were observed in the micelle sample.

To test the ability of SPECT to track the micelles in vivo, ^{111}In-labeled micelles were injected intravenously into athymic nude mice. ^{111}In-labeled free DOTA was also injected to provide a comparison regarding the clearance and circulation time of the micelles. SPECT-CT images were taken 24 hours after the animal treatment, and the resulting images are shown in Figure 3. CT, SPECT, and fused images are shown for both scenarios. Twenty-four hours after the injection of free ^{111}In, the only foci of accumulation are the liver and bladder, which are known sites where small molecules will be filtered. In contrast, the animal which was administered ^{111}In-labeled micelles showed considerable radioactivity throughout the mouse. Two conclusions can be drawn from this result. First, the indium labeling of the micelles was successful and the technique can be used to track the micelles over a longer period of time. Second, intravenously administered micelles circulate for at least 24 hours, which may provide adequate time for both non-specific (passive) and ligand-specific binding to the tumor. Tracking of micelles for longer periods of time will allow for greater detail in tracking micelle accumulation in tumors.

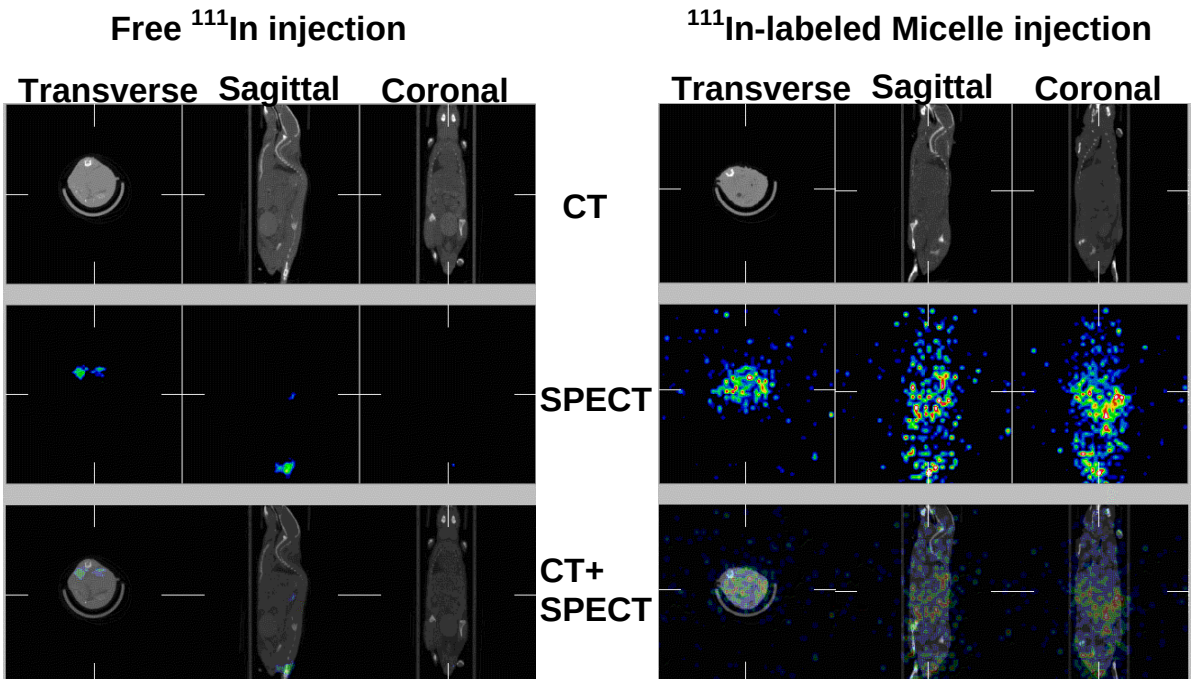

Figure 3. Computed tomography (CT) and single photon emission tomography (SPECT) images of mice injected with free ^{111}In and ^{111}In-labeled micelle. Images were taken 24 h after the injection.

Modifications to stated aims

Modifications to the previously stated aims may be necessary to ensure completion of this project. While CT/SPECT imaging has been successfully utilized to evaluate tissue distribution of polymer micelles, its low spatial resolution (Fig. 3) may become a significant limiting factor to measure tumor targeting efficiency of micelle carriers at cellular or subcellular level. To address this limitation, we propose to use quantum dots (QDs) to label the cRGD-encoded polymer micelles and intravital imaging to examine the vascular targeting efficiency of QD-loaded micelles in a tumor chamber model in athymic nude mice. Correspondingly, fluorescently labeled Annexin V, which is commercially available, can be substituted for ^{99m}Tc-labeled Annexin V. Using *in vivo* fluorescent imaging provides similar capabilities to SPECT imaging with several other advantages. Quantum dots are not radioactive, which allows for more convenient micelle production with no risk of radiation exposure. The lack of a radioactive half-life (versus 2.8 days for ^{111}In) prevents signal decay and allows animal imaging longer after the initial injection. The use of fluorescent tags will also permit live imaging of QD-loaded micelles in the targeting of tumor vasculature in the tumor chamber model. Moreover, microscopic examination of explanted tissues using fluorescence or confocal microscopy for more exact localization of micelles and fluorescent Annexin V. These methodology modifications should provide improvement over the original proposed detection techniques.

Key Research Accomplishments

- Produced doxorubicin-loaded polymer micelles modified with a cRGD targeting ligand.
- Evaluated the uptake of untargeted and cRGD-modified, doxorubicin-containing micelles in cultured cells *in vitro.*
- Produced polymer micelles incorporating both a cRGD targeting ligand and a DOTA moiety for radiolabeling with ^{111}In.
- Performed testing of ^{111}In-radiolabeled micelle distribution with *in vivo* animal studies.

Reportable Outcomes

- Refereed publications
    - Ai, H., C. Flask, B. Weinberg, X.-T. Shuai, M.D. Pagel, D. Farrell, J. Duerk, and J. Gao. "Magnetite-Loaded Polymeric Micelles as Ultrasensitive Magnetic-Resonance Probes." *Advanced Materials* 17, 1949-1952, 2005.
    - Weinberg, B.D., S.J. Schomisch, M. Rahmatalla, W.H. Finlay, A. Chaturvedi, G.R. Wojtkiewicz, and Z. Lee. "Mapping of PET-measured aerosol deposition: a comparison study." *Journal of Aerosol Science* 36, 1157-1176, 2005.
    - Blanco, E., B.D. Weinberg, N.T. Stowe, J.M. Anderson, and J. Gao. "Local release of dexamethasone from polymer millirods effectively prevents fibrosis after radiofrequency ablation." *J Biomed Mater Res A* 76, 174-82, 2006.
    - Weinberg, B.D., H. Ai, E. Blanco, J.M. Anderson, and J. Gao. "Antitumor Efficacy and Local Distribution of Doxorubicin via Intratumoral Delivery from Polymer Millirods." *J Biomed Mater Res A* In Press, 2006.
    - Weinberg, B.D., E. Blanco, S.F. Lempka, J.M. Anderson, A.E. Exner, and J. Gao. "Combined radiofrequency ablation and doxorubicin-eluting polymer implants for liver cancer treatment." *J Biomed Mater Res A* In Press, 2006.
- Book chapter
    - Weinberg, B.D., F. Qian, and J. Gao, "Development and Characterization of Dual-Release Poly(D,L-lactide-co-glycolide) Millirods for Tumor Treatment." *In:* Polymeric Drug Delivery II: Polymeric Matrices and Drug Particle Engineering, S. Svenson, Editor: Oxford University Press. 2006; 169-185.
- Meeting abstracts and presentations
    - Weinberg, B.D., E. Blanco, S.F. Lempka, J.M. Anderson, A.A. Exner, and J. Gao. "Liver tumor treatment with combined radiofrequency ablation and doxorubicin-containing polymer implants," in *Medical Scientist Training Program Winter Retreat, Case Western Reserve University*, February 2006. Cleveland, OH.
    - Weinberg, B.D., E. Blanco, S.F. Lempka, J.M. Anderson, A.A. Exner, and J. Gao. "Liver tumor treatment with combined radiofrequency ablation and doxorubicin-containing polymer implants," in *Research Showcase, Case Western Reserve University*, April 2006. Cleveland, OH.
    - Weinberg, B.D., E. Blanco, S.F. Lempka, J.M. Anderson, A.A. Exner, and J. Gao. "Liver tumor treatment with combined radiofrequency ablation and doxorubicin-containing polymer implants," in *Lepow Research Day, Case Western Reserve University*, May 2006. Cleveland, OH.
- Award
    - Outstanding Poster Award, *Lepow Research Day, Case Western Reserve University*, Cleveland, OH for presentation entitled, "Liver tumor treatment with

combined radiofrequency ablation and doxorubicin-containing polymer implants," May 2006.

Conclusions

In conclusion, doxorubicin-containing micelles for use in treating breast cancer have been fabricated. Micelles including a targeting ligand, cRGD, were taken up into cells more quickly and were more cytotoxic than unlabeled micelles. Micelles were successfully modified to incorporate a chelating ligand, DOTA, which can successfully chelate ^{111}In. ^{111}In-labeled micelles were successfully imaged in mice *in vivo*, where had a prolonged half-life of more than 24 hours. Continuation of this grant will emphasize *in vivo* tracking of micelles and their antitumor effects in a breast cancer model. To obtain the best imaging data, fluorescence imaging methods for micelle detection and apoptosis measurement will be incorporated. The refined imaging techniques will then be used to determine the optimal micelle treatment for breast cancer in a murine model.

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